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The Effects of Aging on Corneal and Ocular Surface Homeostasis in Mice

Manikkuwadura Eranda Harshan De Silva,[1] Lisa J. Hill,[2] Laura E. Downie,[1] and Holly R. Chinnery[1]

[1]Department of Optometry and Vision Sciences, University of Melbourne, Parkville, Victoria, Australia
[2]Neuroscience and Ophthalmology Research Group, Institute of Clinical Sciences, University of Birmingham, Birmingham, United Kingdom

Correspondence: Holly R. Chinnery, Department of Optometry and Vision Sciences, University of Melbourne, Parkville, VIC 3010, Australia; holly.chinnery@unimelb.edu.au.

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Purpose. Aging is a risk factor for dry eye disease. The aim of this study was to investigate if aging is associated with a range of signs of dry eye disease, including tear hyperosmolarity, reduced nerve density, and increased dendritic cell number, in mice.

Method. Healthy C57BL/6 female mice, aged 2 months (young, $n = 10$) and 22 months (aged, $n = 11$) were used. Clinical assessments included corneal sensitivity (Cochet-Bonnet esthesiometry), tear secretion (Phenol red thread test), tear film osmolarity (TearLab osmometer), and corneal thickness (optical coherence tomography). The sum length of the corneal superficial terminals and sub-basal nerves, density of vertical nerve projections, and density and tree area of resident epithelial dendritic cells, were assessed using immunofluorescence and confocal microscopy.

Results. Aged mice had significantly higher tear secretion, lower corneal sensitivity, and a thicker corneal stroma but thinner epithelium. There was no significant intergroup difference for tear osmolarity. Aged mice showed a significantly lower sum length of nerves in the superficial terminals and sub-basal plexus, relative to young mice. Dendritic cell density and morphology were similar in both groups.

Conclusions. In mice, aging is associated with higher tear secretion and corneal epithelial thinning, together with lower corneal nerve density and sensitivity. However, aging was not significantly associated with changes to tear osmolarity or dendritic cell density or size, despite a significant reduction in total nerve length. These findings demonstrate that aged mice exhibit some changes to ocular surface parameters that parallel the anomalies evident in dry eye disease.

Keywords: aging, cornea, sensory nerves, dendritic cells, ocular surface

The cornea is one of the most highly innervated tissues in the body. A rich supply of sensory nerves originates from the ophthalmic branch of the trigeminal nerve and ramifies to form a plexus in the sub-basal region of the corneal epithelium. From the sub-basal plexus, vertically oriented axons project anteriorly, terminating in the superficial epithelium. Corneal nerves are critical for maintaining corneal integrity, through detecting noxious substances, temperature, and regulating wound healing and tear production.[1] However, reduced corneal innervation, due to conditions such as dry eye disease (DED)[2] and diabetes,[3] renders the cornea prone to injury and infection that may lead to vision loss.

Aging is an established risk factor for many of these corneal conditions[4]; however, the mechanism by which the aging process contributes to disease processes remains unclear. Studies are inconclusive with respect to whether aging in humans per se leads to changes in corneal structure and/or function.[5,6] Furthermore, due to the known limitations of in vivo confocal microscopy being only able to image the sub-basal nerve plexus (SBNP),[7] and not the superficial nerve terminals (SNTs), in the corneal epithelium, the effects of aging in these most superficial sensory nerve axons in humans is unclear.[8,9]

By contrast, several rodent studies have demonstrated age-related changes to corneal structure, including an increase in the thickness of Descemet's membrane and a decrease in endothelial cell density.[10,11] The neural architecture has also shown significant changes including a reduction in density in both the SBNP[12–14] and SNTs.[14] Functionally, Stepp et al.[13] demonstrated that mechanical corneal sensitivity in mice decreased with age, which paralleled a reduced density of sub-basal nerve axons, despite no change to SNTs. Interestingly, significant age-related changes to the function of cold-sensing TRPM8$^+$ nociceptors in mouse corneas were recently demonstrated,[15] with low-basal activity TRPM8$^+$ nociceptors exhibiting more unexpected high sensitivity to cold stimulation in aged mice compared with young mice. However, it is unclear whether corneal nerve architecture and distribution correlate with mechanical corneal sensitivity in mice.

Tears play a pivotal role in regulating ocular surface homeostasis, and disruption to physiological tear film dynamics may eventually lead to ocular surface anomalies, such as DED. Tear osmolarity is a marker of tear film health that can be used to evaluate changes in the ocular surface environment, and has been shown to increase with aging in C57BL/6J mice.[15] Interestingly, McClellan et al.[16] also reported an increase in tear volume and accompanying corneal surface irregularities with increasing age in mice.

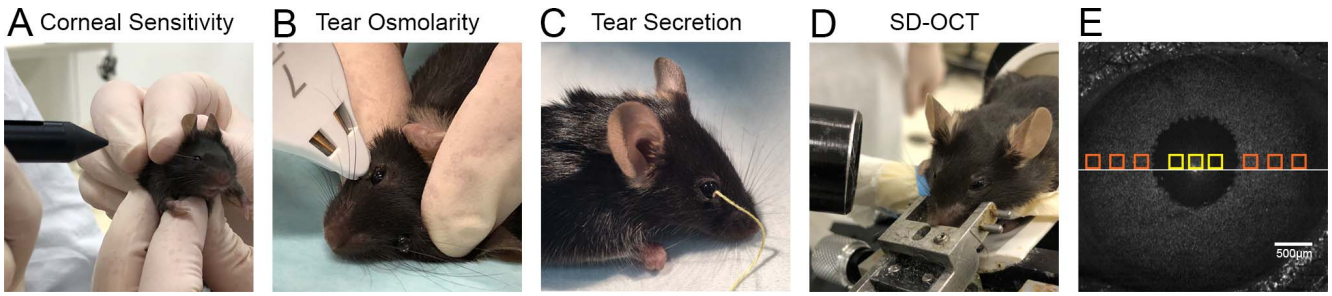

FIGURE 1. Corneal sensitivity was initially assessed using the Cochet-Bonnet esthesiometer (**A**) followed by tear osmolarity using the TearLab osmometer (**B**). Tear secretion was measured using the Phenol red thread test (**C**), and corneal thickness using SD-OCT (**D**). Corneal wholemount immunostaining was performed and confocal images were collected along the diameter of the cornea (**E**). Four to six scans were collected in the periphery (*orange*) and approximately two to four scans were collected in the central cornea (*yellow*).

However, there remains inconsistency with respect to the reported impact of aging on other ocular surface parameters, such as corneal thickness, where some studies have reported no age-related changes,[17,18] and others have shown an increased thickness compared with young mice.[19] In addition, the effects of aging on corneal immune responses, in particular the number, distribution, and morphology of resident epithelial dendritic cells (DCs), is unclear. The normal corneal epithelium has a resident population of DCs that have been well characterized in both humans and rodents.[20–24] Dendritic cells predominantly reside in the basal epithelium, where they serve not only as immune sentinels, surveying the tissue for pathogens and other foreign bodies, but also act as a bridge between the innate and adaptive immune systems.[25] Most DCs in the corneal epithelium are located in the peripheral region, with a decline in numbers centrally. A smaller population of DCs also exist in the corneal stroma.[26,27] For clarity, reference to DCs in this present study will relate only to corneal epithelial DCs.

Ocular pathologies, such as DED[28] and glaucoma,[29] are associated with an increase in corneal DC density. Along with corneal epithelial cells, DCs also have been implicated in corneal wound healing responses.[21] Recent mouse studies have demonstrated that DCs and corneal nerves form physical contacts within the SBNP, suggesting a direct interaction between the immune system and peripheral nerves.[27] Furthermore, pharmacological depletion of the resident DC population has been shown to be correlated with a decline in corneal nerve structure under homeostatic conditions.[27] This is in contrast to the reported negative correlation between DC density and nerve density in a range of corneal pathologies caused by infectious keratitis.[30,31] However, whether there is a change in the neuroimmune interaction or in the density and morphology of DCs in the aging mouse cornea is still unclear.

The aim of this cross-sectional study was to comprehensively investigate the impact of aging on key ocular surface parameters in mice, by combining both in vivo clinical assessments (including tear osmolarity, tear secretion, corneal sensitivity, and corneal thickness) and ex vivo immunohistochemical techniques to quantify corneal nerve architecture and the density of resident epithelial DCs. Correlation analyses were performed to assess for potential associations between clinical and ex vivo measurements.

METHODS

Animals

Female C57BL/6 mice, aged 2 months (young; $n = 10$), and retired breeders, aged 22 months (aged; $n = 11$), were housed under pathogen-free conditions at the Florey Institute of Neuroscience and Mental Health, Victoria, Australia. Only female mice were used due to the known increased prevalence of DED in female human patients.[32] Sample size calculations were informed based on a previous similar study by Wang et al.,[14] who detected statistically significant differences in nerve density in mice aged 2 months and 15 months ($n = 6$ per group). With regard to corneal dendritic cell differences, we were unable to collect pilot data from age-matched mice, so instead considered group sizes used by McClellan et al.,[16] who compared immunohistochemically stained conjunctival sections to measure $CD4^+$ T-cell density in young (i.e., 2 months) and aged (i.e., 24 months) mice. In their study, group sizes of $n = 5$ were adequate to detect an approximate 2-fold increase in $CD4^+$ T cells in the epithelium of aged female mice. The mice underwent in vivo clinical assessments to quantify corneal sensitivity, tear osmolarity, tear secretion, and corneal thickness, and ex vivo assessments using wholemount immunofluorescence to quantify corneal nerve architecture and length, and DC density and morphology. All study procedures were approved by the Animal Ethics Committee at the Florey Institute of Neuroscience and Mental Health and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Clinical Assessments

Corneal Sensation. A Cochet-Bonnet esthesiometer (Luneau SAS #10129; Prunay-le-Gillon, France), with a 0.12-mm diameter nylon filament, was used to assess corneal sensitivity (Fig. 1A). Corneal sensitivity thresholds were measured in the left eye by the same examiner. The procedure involved gently touching the tip of the nylon filament onto the cornea, perpendicular to the corneal apex. A blink response, measured by two independent observers, was considered to indicate detection of the mechanical stimulus (i.e., a positive sensation response). Measurements were made at the recommended starting length of 60 mm (corresponding to the lowest mechanical threshold, and thus highest corneal sensitivity), and gradually decreased in 5-mm steps. For each filament length, 10 repeat trials were performed. If a positive sensation response was elicited for fewer than 5 of the 10 trials, the filament length was decreased and another 10 trials were repeated at this new length. This process was repeated until positive sensation responses were achieved for 5 or more of the 10 repeat trials, and this value was recorded as the corneal sensitivity threshold.

Tear Osmolarity. Mice were deeply anesthetized by intraperitoneal injection (ketamine 80 mg/kg and xylazine 10 mg/kg; Troy Laboratories, Glendenning, NSW, Australia) and tear osmolarity was assessed in the right eye using the TearLab osmometer (TearLab Corp., San Diego, CA, USA) (Fig. 1B),

which was calibrated daily according to the manufacturer's instructions. Room temperature was 21°C when measurements were performed.[33] The same diagnostic pen was used for all measurements, which were collected on the same day.

Tear Secretion. Immediately after measuring tear osmolarity, tear secretion was assessed in the right eye, using the Phenol red thread test (Tianjin Jingming New Technological Development Co Ltd., Tianjin, China) (Fig. 1C). The edge of the thread was gently hooked over the lower eyelid and left in place for 20 seconds. The wetted length (in millimeters), indicated by a color change from yellow to red, was immediately measured.

Corneal Thickness. Anterior segment images were noninvasively captured using spectral-domain optical coherence tomography (SD-OCT) (Envisu R2200 VHR; Bioptigen Inc., Durham, NC, USA) (Fig. 1D). Anesthetized mice were placed on an animal imaging mount and rodent alignment stage (AIM-RAS), and the anterior segment of the eye was imaged using an 18-mm telecentric objective lens to capture a transverse plane of the cornea to quantify the central corneal thickness. Saline eye drops were periodically administered to both eyes in order to maintain corneal hydration. Volumetric 4 × 4-mm scans were performed.

Corneal Wholemount Immunostaining. Eyes were fixed in 2% paraformaldehyde/0.02% picric acid in 0.1 M sodium phosphate buffer for 15 minutes, then corneas were dissected and post-fixed for an additional 24 hours at 4°C. The samples were washed three times with 0.1M PBS containing 0.1% bovine serum albumin (PBS-BSA), to remove the fixation media, followed by incubation in prewarmed 0.5M EDTA (37°C for 45 minutes). To block nonspecific binding, tissues were incubated in PBS containing 10% normal goat serum plus 0.3% Triton X-100 solution for 60 minutes at room temperature, on an agitating platform. The tissue was then incubated in primary antibody rabbit βIII-tubulin antibody (1:1000, #T2200; Sigma-Aldrich, St. Louis, MO, USA) and rat anti-mouse MHC Class II antibody (1:1000, #336999; BD Biosciences, Franklin Lakes, NJ, USA) for 72 hours at 4°C. Samples were washed three times then incubated with secondary antibodies goat anti-rat Cy3 (1:1000, #10522, Invitrogen, Carlsbad, CA, USA), goat anti-rabbit 647 (1:1000, #21244; LifeTechnologies, Carlsbad, CA, USA), and Hoechst 33342 (1:1000) for 3 hours at room temperature. Tissues were cover-slipped with the epithelium oriented anteriorly.

Confocal Imaging. Immunostained wholemount corneas were imaged using a Leica SP8 inverted confocal microscope (DMI6000; Leica Microsystems, Buffalo Grove, IL, USA). A ×40 objective lens (1.30NA oil) was used to perform a Z-stack scan from the superficial epithelium to the anterior stroma (0.35-μm step size; 1024 × 1024 pixel resolution; 290 × 290 μm). Two to four scans were collected within the 1-mm central cornea diameter, and four to six scans were collected in within the 1-mm to 2-mm peripheral cornea (Fig. 1E).

Image Analyses

Corneal Thickness. Central corneal thickness was quantified at the apex, in the right eye, in all mice. Using the volumetric SD-OCT anterior segment scans, the en face image and corresponding transverse images were used to determine the corneal apex image frame (denoted as C_o). The images of two frames (C_{o+2}) and four frames (C_{o+4}) superior to the C_o reference point were identified. This process was repeated inferiorly (to define C_{o-2} and C_{o-4}, respectively). These five images were analysed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). The "Straight Segment" feature in ImageJ was used to quantify the epithelial, stromal thickness, and total thickness. The mean value for the five images (C_o, C_{o+2}, C_{o+4}, C_{o-2}, and C_{o-4}) for each of these parameters was used as a representative value for each mouse.

Corneal Nerves. Before nerve image analysis, confocal Z-projections were generated for the SBNP (Figs. 2A, 2B), vertical nerve projections (Figs. 2C, 2D), and SNTs (Figs. 2E, 2F). To achieve a consistent number of frames to analyze the SNTs, and to avoid images with high background autofluorescence, nine consecutive frames (3.15 μm total Z thickness) from the superficial epithelium were stacked. To measure the number of axons projecting vertically from the SBNP to the superficial epithelium, a single frame containing only cross-sectional views of the vertical axons was exported. The density of the vertical nerve projections was counted using the cell counter plugin of ImageJ (measured as number/mm^2). For the SBNP and the SNTs, the sum length (measured as the total nerve length, in microns) was determined using the nerve tracing plugin of ImageJ, NeuronJ (Fig. 2). All image file names were masked by an independent researcher and then analyzed by another independent researcher. After data analysis was complete, images were unmasked and data were analyzed separately for the central and peripheral corneal regions.

Dendritic Cell Density and Tree Area. For each corneal flatmount, images of MHC Class II positive DCs were captured in the central (one image) and peripheral cornea (three images) using a ×10 objective (900 × 673 μm; BX51, Olympus, Tokyo, Japan). DCs were counted using the cell counter plugin (ImageJ) and DC field area (dendrite tip to tip) was assessed using the "Polygon Selections" feature tool in ImageJ.[22] Each cell from the central cornea was included in the analysis, whereas only 10 DCs from the peripheral corneas were included, as the DC density in the peripheral is significantly greater than the central cornea. The selection of the 10 peripheral DCs was performed in a random manner, with the first 10 cells analyzed, starting from the top left hand corner of the image.

Statistical Analyses

Statistical analyses were performed in GraphPad Prism (version 7.0; GraphPad Software, Inc., La Jolla, CA, USA). Data normality was tested using the D'Agostino and Pearson omnibus test and outliers were determined using the Robust regression and Outlier test with a coefficient Q-value of 1%. Unpaired Student *t*-tests were performed to compare young and aged mice for clinical parameters (corneal sensitivity, tear secretion, and corneal thickness) and to compare the corneal cellular characteristics (DC morphology and density, and nerve density and vertical nerve projection in the central and peripheral cornea of young and aged mice). To assess relationships between parameters, Pearson's correlation coefficient was used, except for corneal sensitivity, which was performed using Spearman's correlation coefficient. All data are presented as mean ± SEM. For statistical significance, the alpha value was set at 0.05.

Results

Aged Mice Have Higher Tear Secretion Than Young Mice, However There Is No Difference in Tear Osmolarity

Corneal sensitivity, tear osmolarity, and tear secretion were compared between young and aged mice. Corneal sensitivity (Fig. 3A, young: 5.5 ± 0 versus aged: 4.85 ± 0.3 mm, $P =$

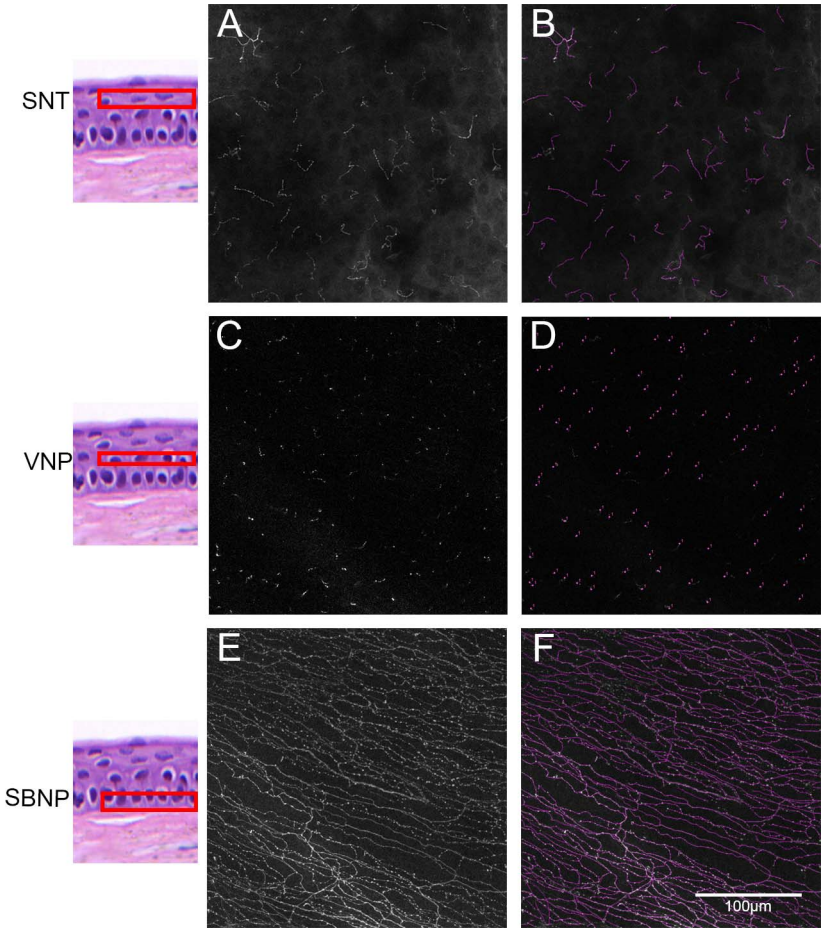

FIGURE 2. Representative en face confocal images and corresponding Neuron J tracings of the SNTs (**A**, **B**), vertical nerve projections (VNPs; **C**, **D**), and SBNP (**E**, **F**). *Red outline* in the hematoxylin and eosin–stained images show the approximate location of the corresponding confocal layers.

0.032) showed a modest but statistically significant intergroup difference, whereas tear osmolarity (Fig. 3B, young: 346.3 ± 16.5 versus aged: 366.8 ± 7.8 mOsmol/L, $P = 0.23$) did not show any significant difference. Aged mice also had higher tear secretion than young mice (Fig. 3C, young: 2.85 ± 0.4 versus aged: 11.23 ± 2.0 mm, $P = 0.0009$). When normalized to body weight, tear secretion in aged mice remained significantly greater (young: 0.16 ± 0.06 mm/g versus aged: 0.35 ± 0.22 mm/g, $P = 0.01$, Supplementary Fig. S1).

Aged Mice Have a Thicker Corneal Stroma But Thinner Corneal Epithelium

Representative SD-OCT scans of the cornea are shown for young and aged mice in Figure 4A. Central corneal thickness was greater in aged mice (Fig. 4B, young: 83.7 ± 1.55 μm versus aged: 92.4 ± 3.6 μm, $P = 0.04$). Considering the component layers of the cornea, corneal stromal thickness was greater in aged than young mice (Fig. 4B, young: 49.7 ± 1.4 versus aged: 63.4 ± 3.5 μm, $P = 0.004$). In contrast, the

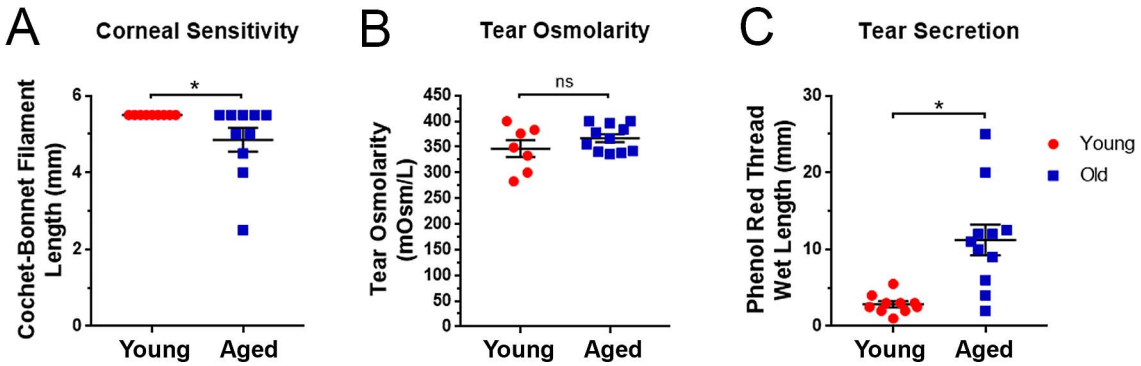

FIGURE 3. Reduced corneal sensitivity of aged mice compared with young mice (**A**). No difference in tear osmolarity of young mice compared with aged mice (**B**). Tear secretion was elevated in aged mice as measured using a Phenol red thread test (**C**). For statistical significance, $^*P < 0.05$.

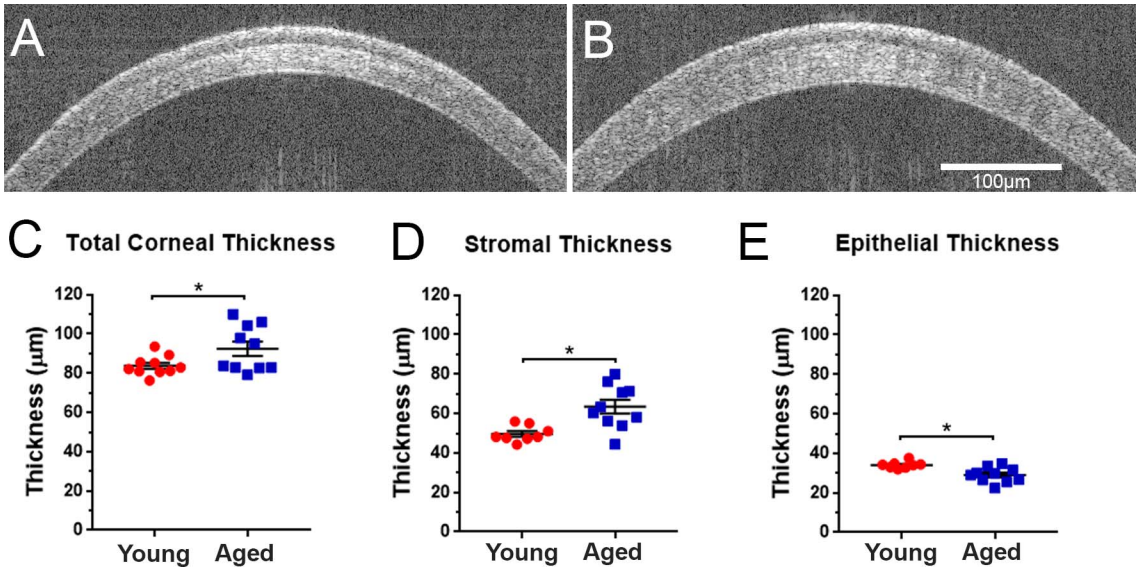

FIGURE 4. Representative transverse SD-OCT images of the central cornea of young (**A**) and aged mice (**B**). Comparison of the total corneal thickness (**C**), stromal thickness (**D**), and epithelial thickness (**E**) of young and aged mice. For statistical significance, $^*P < 0.05$.

epithelium was significantly thicker in young mice (Fig. 4B, young: 34.1 ± 0.6 versus aged: 29.0 ± 1.2 µm, $P = 0.003$).

Corneal Nerve Sum Length in the Superficial Epithelium and Sub-Basal Region, and Vertical Nerve Projection Density Are Significantly Lower in Aged Mice

The sum length of the SNTs was significantly higher in young mice in both the central (Figs. 5A–E, young: 2536 ± 148.1 versus aged: 1276 ± 180.3 µm, $P < 0.0001$) and peripheral regions (Figs. 5A–E, young: 1919 ± 115.2 versus aged: 1100 ± 143.7 µm, $P = 0.0003$). A similar effect was observed for the SBNP sum length, with young mice showing a significantly higher sum nerve length than aged mice in both the central (Figs. 5K–O, young: 10980 ± 654.9 versus aged: 9086 ± 574 µm, $P = 0.04$) and peripheral cornea (Figs. 5K–O, young: 11031 ± 402.2 versus aged: 7297 ± 498.4 µm, $P < 0.0001$). Past studies have demonstrated that SNT and SBNP lengths may change under different ocular surface conditions; however, we sought to determine whether there was a change to the number of axons supplying the terminals.[34,35] Vertical nerve projections that stemmed from the SBNP toward the apical epithelium were significantly higher in young mice, compared with aged mice, in the central (Figs. 5F–J, young: 446.4 ± 58.9 versus aged: 225.9 ± 38.4 number/mm^2, $P = 0.005$) and peripheral cornea (Figs. 5F–J, young: 398.6 ± 34.9 versus aged: 172.0 ± 23.9 number/mm^2, $P < 0.0001$).

Corneal Dendritic Cell Density and Tree Area Are Similar in Young and Aged Mice

The dendritic tree area[22] is used as an indicator of DC maturity in the corneal epithelium.[36] MHC Class II positive cells were visible in the central (Figs. 6A, 6B) and peripheral (Figs. 6C, 6D) corneal epithelium. There was no significant difference in DC density between young and aged mice in either the central (young: 2.6 ± 0.7 versus aged: 3.8 ± 1.2 cells/mm^2, $P = 0.37$, Fig. 6E) or peripheral cornea (young: 28.8 ± 1.6 versus aged: 31.8 ± 3.7 cells/mm^2; $P = 0.48$, Fig. 6F). When comparing the size of MHC Class II positive DCs in the corneal epithelium, there was no significant intergroup difference in average DC tree area in either the central (Fig. 6G, young: 2050 ± 27 versus aged: 1551 ± 272 µm^2, $P = 0.18$) or peripheral cornea (Fig. 6H, young: 2165 ± 111 versus aged: 2411 ± 184 µm^2, $P = 0.28$).

Correlations Among Corneal Nerve Architecture, Dendritic Cells, and Clinical Parameters

The Table summarizes the relationships between ex vivo corneal nerve parameters and in vivo clinical parameters. There was a significant negative correlation between the SNT density and tear secretion in the central cornea ($r = –0.49$; $P = 0.02$) but not in the periphery ($r = –0.43$; $P = 0.058$). There was also a significant negative correlation between the SBNP density and tear secretion in the peripheral ($r = –0.65$; $P = 0.0057$), but not the central ($r = –0.27$; $P = 0.42$), cornea. A negative correlation was observed between central and peripheral corneal DC density and corneal sensitivity, whereas a positive correlation was evident between peripheral DC density and tear volume ($r = 0.52$; $P = 0.018$). There were no significant associations between DC density and nerve density in the central or peripheral corneal regions; however, there was a statistically significant negative association between central DC density and peripheral SBNP lengths ($r = –0.549$; $P = 0.01$).

DISCUSSION

The current study investigated the effects of aging on the ocular surface of C57BL/6 mice. We measured clinical parameters, including corneal sensitivity, tear osmolarity, tear volume, and corneal thickness, in combination with the ex vivo measurement of corneal nerves and resident dendritic cells. At 22 months, a C57BL/6 mouse is age-equivalent to an approximately 65-year-old human, which may provide key insights into how aging influences the ocular surface in humans.[37] A number of other laboratories have reported age-related changes to the ocular surface of C57BL/6J mice,

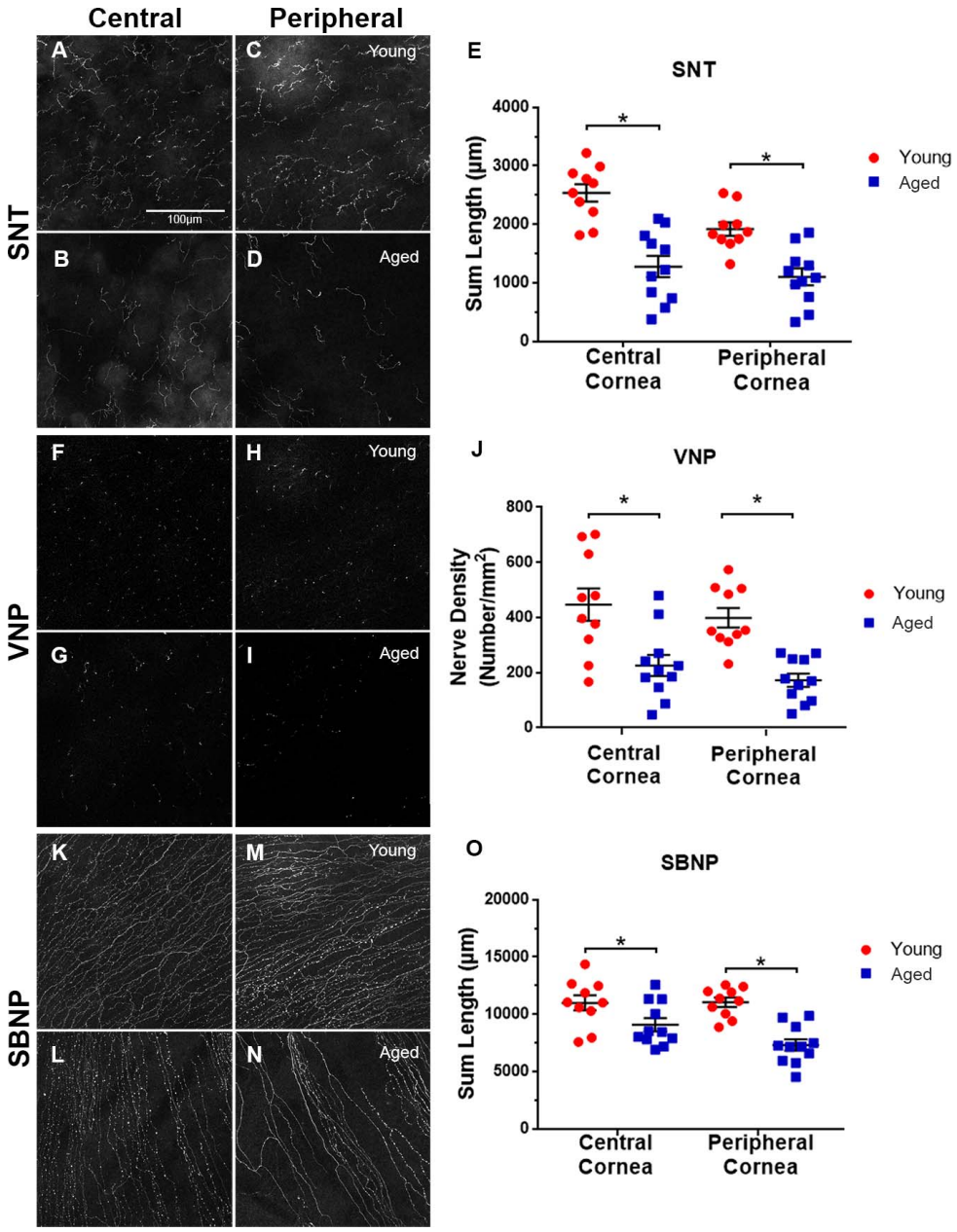

FIGURE 5. Representative images of the SNT in the cornea of young (**A**, central, **C**, peripheral) and aged mice (**B**, central, **D**, peripheral) and corresponding quantitative analysis of the sum length of nerves (**E**). Comparison of the VNP density (**F**–**J**) and SBNP sum length (**K**–**O**) between young and aged mice in the central and peripheral cornea, demonstrating a lower density and sum length in both corneal regions in aged mice compared with young mice. For statistical significance, $^{*}P < 0.05$.

including corneal sensitivity, nerve density, and surface irregularity[13]; tear volume, surface irregularity, goblet cell changes, and $CD4^{+}$ T-cell accumulation in the conjunctiva[16]; and tear volume, osmolarity, and alterations in $TRPM8^{+}$ cold-sensing terminals.[15] Our study adds to these previous studies by examining correlations between quantitative clinical parameters (including corneal thickness, corneal sensitivity, tear osmolarity and tear volume) with ex vivo measures of corneal nerve density and corneal epithelial dendritic cell number and size in healthy aged female mice.

Interpretation of the sensory nerve data from the present study suggest that aged mice have a lower nerve sum length in both the apical (SNT) and basal layers (SBNP) of the epithelium, as well as a lower density of vertically projecting nerve axons. Both the central and peripheral cornea showed this effect for all nerves within the epithelial layer, indicating a global effect of aging on corneal nerve architecture in mice. Our findings corroborate other studies that have explored age-related changes in ex vivo corneal nerves using immunohistochemical methods in similarly aged C57BL6/J mice (i.e., 24 months),[14] in 12-month-old animals,[12] and in 15-month-old C57BL/6J mice.[14] Stepp et al.[13] also reported that although aging led to a significant reduction in SBNP axon density, the basal extensions projecting toward the SNTs were significantly increased. One possible explanation for this disparity between studies is the method used to quantify basal extensions. Stepp

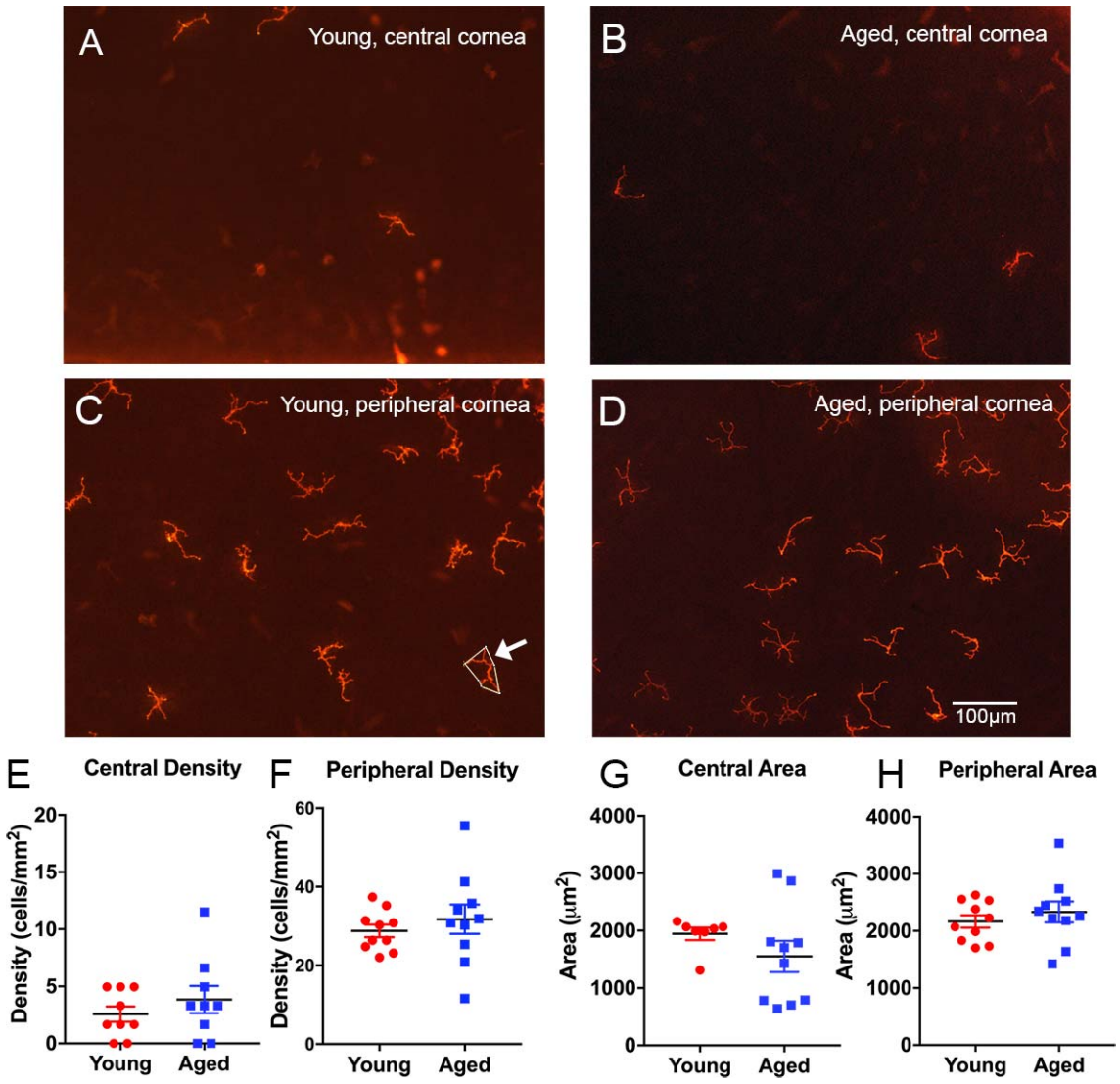

FIGURE 6. Representative images of MHC Class II positive DCs in the central (**A**, **B**) and peripheral corneal epithelium (**C**, **D**). Example of the tracing of a dendritic cell tree area using the "Polygon selections" function of ImageJ (*arrow*, **C**). There were no statistically significant differences in the mean tree area of dendritic cells in the central (**E**, **F**) or peripheral (**G**, **H**) cornea of young and aged mice. For statistical significance, $^{*}P < 0.05$.

and colleagues[13] measured nerve terminal extensions (pixel intensity) in Z-projected images of smaller fields of view (135 × 135 μm). In the current study, we traced the sum length of nerves in en face images that were 290 × 290 μm, from nine nonoverlapping areas per cornea, and quantified the number of vertically projecting axons from the same en face image sequences. Interestingly, Dvorscak and Marfurt[38] showed that although SNT density decreased with age in rats, there was an increase in the SBNP density, which was hypothesized to be due an increased growth of the SBNP as a compensatory mechanism for the decrease in the SNT. A further possible explanation for the discrepancy in nerve density data may be due to differences in animal housing environments, species used, or the signal intensity of nerve immunostaining results.[13,39] Human studies have also shown substantial heterogeneity, with some studies reporting the nerve density in the SBNP to decrease,[40] or have no change[8] with age. However, this may be due to variations in the instrumentation used to capture the image, such as the Heidelberg Retina Tomograph with the Rostock Cornea Module or Confoscan 4-slit scanning confocal microscope, the number of images assessed, and the corneal location, such as the central or peripheral cornea.[7,14]

A number of human and mouse studies have established a structural relationship between dendritic cells and corneal nerves, with current hypotheses suggesting that elevated DC density leads to changes in the corneal nerves, and subsequently, a disruption to corneal homeostasis.[27,41] Similar to our findings in mice, human studies have identified no significant change to corneal epithelial DCs with aging in a healthy population.[42] In the context of inflammation, numerous studies have highlighted a negative correlation between corneal nerves and DC density, suggesting a functional relationship between corneal nerve pathology and DC activation in the corneal epithelium.[27,41] However, the number and morphology of corneal epithelial DCs in aging mice has not been explored until now. The present study found no significant difference in DC density or morphology between young and aged mice, despite a significant reduction in nerve density in aged mice. A recent study using young mice

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TABLE. Relationship Between In Vivo Clinical and Ex Vivo Parameters

	SNT (Central)	SNT (Peripheral)	SBNP (Central)	SBNP (Peripheral)	Corneal Sensitivity	Tear Osmolarity	Tear Volume	Corneal Thickness	Central DC Density	Peripheral DC Density
SNT (central)	1									
SNT (peripheral)	0.808*	1								
SBNP (central)	0.489*	0.564*	1							
SBNP (peripheral)	0.581*	0.484*	0.322	1						
Corneal sensitivity	0.437	0.519*	0.099	0.562*	1					
Tear osmolarity	−0.329	−0.355	0.151	−0.208	0.172	1				
Tear volume	−0.487*	−0.430	−0.271	−0.655†	−0.602*	0.174	1			
Corneal thickness	0.170	−0.034	−0.020	0.144	−0.123	0.132	−0.222	1		
Central DC density	0.001	0.041	0.191	−0.549*	−0.606*	−0.239	0.204	−0.164	1	
Peripheral DC density	−0.215	−0.183	0.139	−0.307	−0.550*	0.214	0.523*	0.218	0.252	1

All relationships were assessed using Pearson's test excluding corneal sensitivity which was performed by Spearman's correlation coefficient.

* Statistically significant ($P < 0.05$) correlation.

† Statistically significant ($P < 0.01$) correlation.

demonstrated a physical interaction between DCs and corneal nerves in normal homeostasis and after injury.[27] Studies exploring the effect of comorbid conditions, such as diabetes, on the neuroimmune interaction of the cornea in mice have shown that DC density increased while nerve fiber density decreased.[43] The lack of correlation between DC density and corneal nerve density in the current study is interesting, and indicates that age-related changes to corneal nerves occurs independent of changes to DC numbers. Some studies exploring dendritic cell function have highlighted that the activation and initiation of the adaptive immune system is impaired with aging,[44] whereas other studies have shown it to be preserved.[45] Future research could explore whether aging alters DC activation and function in healthy mouse corneas.

Furthermore, in both human and mouse studies of DED, DC changes have been implicated in the corneal epithelium, with increased density of enlarged DCs in humans[41] and upregulation of activation markers in mouse epithelial dendritic cells.[46] Despite several ocular surface changes that are similar to DED pathology observed in the present study, such as decreased nerve density, epithelial thinning, and increased tear production, the unaffected DC morphology and number, along with unchanged tear osmolarity, may suggest that a relatively stable ocular surface immune status is maintained in aged female mice.

Tear secretion plays a pivotal role in keeping the ocular surface hydrated, lubricated, and clear of debris. In studies of lacrimal and meibomian glands in mice, age-related changes such as periductal fibrosis, acinar cell atrophy, and lymphocytic infiltration were reported.[47–49] The outermost lipid layer plays a crucial role in regulating tear film evaporation from the ocular surface.[50] Therefore, a reduction in lipid layer integrity with age could adversely affect tear volume. However, similar to our findings, other studies assessing tear volume in mice also found that aged mice produce more tears compared with younger mice, as measured using the Phenol red thread test.[15,16,51] A possible mechanism underlying this finding could relate to changes in the structure of the nasolacrimal ducts leading to a lower rate of tear drainage.[52] Although human studies have identified that tear drainage decreases with age,[53] future research should explore this paradoxical phenomenon in mice.

A further consideration is that aqueous tear secretion is driven by corneal nerve stimulation.[54] Activation of corneal afferent neurons due to environmental changes increases basal tear secretion. However paradoxically, our findings suggest that increased tear secretion is correlated with a decrease in nerve sum length in both the SBNP and SNT of the central and peripheral cornea. Mouse studies have shown that a subpopulation of polymodal corneal nerves predominantly express either TRPM8$^+$ or TRPV1 nociceptors.[55] However, the TRPM8$^+$ corneal nerves are responsible for detecting corneal surface cooling, and are predominantly involved in basal tearing regulation.[56] These cold-sensing neurons have recently been shown to be affected, both structurally and functionally, during aging,[15] with low-threshold TRPM8$^+$ cold receptor neurons decreasing with age, along with modified responses in high-threshold cold receptor neurons.[15] The higher tear secretion in aged mice observed in the current study may thus may be related to a reduction in the TRPM8-specific neural signals, along with changes in the nasolacrimal system.

Exploring the impact of aging on tear osmolarity is of growing interest, as it provides clinical insight into the mechanisms underpinning age-related conditions, such as DED. Despite tear osmolarity being slightly higher in the aged mice, this difference was not statistically significant. Interestingly, our tear osmolarity values in young and aged mice were similar to those reported by Alcalde et al.[15]; however, their

findings showed significant differences between the age groups, which might be explained by the inclusion of both eyes in the analysis as independent samples ($n = 10$–11 eyes/group in our study versus $n = 19$–20 eyes per group in the Alcalde et al. study).[16] Stewart et al.[57] also reported that young C57BL/6 mice had a significantly lower tear osmolarity compared with the values in our study. This apparent difference may be explained by the different methodologies used to measure tear osmolarity. Although the use of the TearLab osmometer to measure tear osmolarity in mice has been reported in several studies,[15,58,59] the mean baseline values are generally higher compared with typical readings from healthy human tear samples.[60] It is unclear whether this discrepancy is due to true differences in homeostatic tear osmolarity in mice compared with humans, or due to technical limitations in using this device to reliably measure tear osmolarity in small animals. The observed increase in tear secretion in our study, together with a stable tear osmolarity, may indicate that tear solute concentrations are relatively stable through the life span of mice but that adequate tear drainage may be compromised. A limitation of this study is the absence of tear protein analysis, including proinflammatory cytokines. Although such an analysis could have provided further insight into age-related changes in tear fluid composition in mice, it was beyond the scope of the current study.

Consistent with a lower sum nerve length in all corneal epithelial layers and corneal regions, mechanical sensitivity was significantly different in aged mice compared with young mice. Interestingly, we found a significant correlation between nerve length and sensitivity only in the peripheral cornea, and not the central cornea. These findings are consistent with the sensitivity measurements reported by Stepp et al.,[13] who also demonstrated that central corneal mechanical sensitivity decreased in aged mice. Interestingly, recent findings by Alcalde et al.[15] suggest that a reduction in cold-sensitive nerves in the aging mouse cornea was not associated with loss of function. Rather, the remaining cold-sensing nociceptors demonstrated threshold responses similar to or greater than young mice, suggesting a differential reduction in functional subsets of sensory nerves in aging.[15]

We used SD-OCT to evaluate corneal structure and found a greater corneal stromal thickness but lower epithelial thickness in aged mice. However, there was no correlation between corneal thickness and other clinical parameters, such as tear volume and osmolarity, and nerve architecture. It is well accepted that the density of corneal endothelial cells decreases with aging, along with a compensatory increase in cell size (polymegathism).[11,61,62] Thus, although endothelial cell density was not quantified in the current study, it is likely that the observed increase in stromal thickness could be explained by an age-related decline in endothelial cell density. Inomata et al.[19] also reported an increase in the corneal thickness, but no change to epithelial thickness, of BALB/c mice at 14 months of age. This disparity may be accounted for by the genetic variation between different mouse strains and different ages.

Conclusions

We demonstrated that aging has distinct effects on specific ocular surface elements, including tear volume, and corneal sensitivity, nerve density and thickness, but no significant effect on other parameters, such as corneal DC density and tear osmolarity. We also found that the significant decline in nerve density in the mouse cornea is not associated with changes to DC morphology, distribution, or density throughout the central and peripheral cornea, suggesting a preservation of corneal immune cell number and morphology in normal aged mice.

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